The Sweet and Sorrow of Pancreatic cancer

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English Summary

For half a century, the incidence and mortality of patients with pancreatic cancer has hardly changed [1-4]. Due to increased accuracy in diagnosis [5, 6], mortality in pancreatic cancer even has increased in the industrialized world [7]. The rationale behind these dismal facts is not clearly understood [7]. In this thesis I focused mainly on the highly malignant pancreatic ductal adenocarcinoma (PDAC) which accounts for approximately 90% of pancreatic tumours [8].

The major recognized risk factor for PDAC is tobacco smoking, others are overweight, obesity and overweight related diabetes type II, pancreatitis [9] and heavy alcohol consumption [6, 10, 11]. Symptoms at early stage are nonexistent or often vague and easily confused with other diseases [6, 12].

PDAC has a propensity to form early metastases and to gain high resistance to both chemo- and radiotherapy [8]. The word metastasis is based on two Greek words meaning "displacement", from μετά, (meta) and "placement" from στάσις (stasis). Therefore metastasis is the spread of tumours, from one place in the body, to another location not directly connected with it to form secondary tumours. The most common sites for metastasis formation in PDAC are the lymph nodes and liver, followed by the peritoneum, lungs, bones and adrenal glands [12]. However, metastatic PDAC can be found in almost all organs of the body [13]. Surgical removal of the cancer gives a prospect on survival, but this is possible only for 15–20% of the patients [14], whereas gemcitabine is the standard and most efficient therapy for metastatic PDAC [15, 16]. Unfortunately, there is no reliable screening test or specific diagnostic markers for early detection of PDAC. Therefore, more fundamental knowledge of PDAC development and biology is urgently needed to facilitate novel approaches for diagnosis and treatment of PDAC.

In recent years, the essential contribution of changes in protein-glycosylation to progression of cancer has been established in many different cancer types [17-21]. In this thesis, I focus on the role of galectin-4, a glycan-binding protein, and tumour-glycosylation in PDAC, with the aim to increase our understanding of cancer progression in particular in PDAC, and to promote approaches to discover novel tools for early detection and diagnosis of PDAC.

Glycans are highly branched sugar structures composed of different building blocks, the monosaccharides. Glucose (Glc) and galactose (Gal) are well known monosaccharides in daily life, but other monosaccharides include mannose (Man); *N*-acetylglucosamine (GlcNAc); *N*-acetylgalactosamine (GalNAc); glucuronic acid (GlcA); fucose (Fuc); xylose (Xyl) and sialic acid (Sia). They are mostly attached by enzymes (glycosyltransferases) to proteins (thus forming glycoproteins) or lipids (glycolipids) on the surface of cells. The way these glycans are attached to proteins or lipids determines their classification. For example, *N*-glycans and *O*-glycans are connected to proteins via NH2 and OH-groups of a protein, respectively. There are also other types of glycans that are attached to other types of carriers, such as glycosaminoglycans (GAGs) which are linked to extracellular matrix proteins, thereby forming proteoglycans, glycosphingolipids consisting of a mono/oligosaccharide linked to a ceramide and glycosylphosphatidylinositol (GPI)-linked proteins, which are anchored at the outer layer of the cell's plasma membrane.

Glycosylation is one of the most common post-translational modifications of proteins, i.e., alterations of proteins after its synthesis within the cell. In eukaryotes, approximately 50% of all proteins are assumed to be glycosylated. Glycosylation is a complex and highly regulated process, requiring the concerted action of more than 100 enzymes (glycosyltransferases), including the enzymes involved in biosynthesis pathways of *N*- and *O*-glycans [22]. In addition, alterations of glycan structures or/and aberrant glycosylation of glycoproteins can induce crucial alterations in the tumour cell, or its environment, leading to cancer thriving, such as by immune evasion [17, 23, 24], modification of signalling pathways [25, 26] or increased cell migration [27-30]. Importantly, protein levels of some of the glycosyltransferases have been shown to be altered under low oxygen levels (hypoxia) [31].

Glycan structures are in general recognized by specific glycan-binding proteins (lectins). These include for example C-type lectins on the surface of immune cells and galectins, a family of soluble lectins. A correct glycan-lectin interaction is often crucial for the regulation of 'glycan-dependent' cellular functions. In addition to aberrant glycosylation, the expression of lectins can be disturbed in cancer, in either case leading to changes in glycan-lectin interactions and consequently, cellular functions. Galectins are galactose-binding proteins that can be divided into 3 different classes: prototype galectins with one carbohydrate recognition domain (CRD) which form homodimers; tandem-repeat galectins which contain 2 distinct CRDs in tandem connected by a linker peptide; and the chimera-type Galectin-3, which consists of short stretches of proline and glycine-rich tandem repeats fused to the CRD.

In this thesis, I portray the expression of glycans in pancreatic cancer and colorectal cancer (CRC), in rela-

tion to their immune-recognition by C-type lectins, and the function of the soluble tandem-repeat lectin galectin-4 (Gal-4) in migration and metastasis of PDAC cells.

1. Galectin-4 : Friend or foe?

The aberrant expression of Gal-4 could disturb normal cell function during carcinogenesis. To evaluate the possibility to use Gal-4 as a diagnostic marker and/or therapeutic target a deeper understanding of the role of Gal-4 in cancer is necessary.

In healthy individuals Gal-4 is selectively expressed in the gastrointestinal tract [32-35], neurons [36-38] and cells that provide support and protection to neurons, oligodendrocytes [39]. In several cancer types, Gal-4 expression is increased at the messenger RNA (mRNA) and protein level and even higher at the malignant stages of carcinomas, including PDAC tissues [40-44] and in patients' blood serum [41, 45]. This indicates that Gal-4 could be a pro-cancer factor. However, in colorectal cancer (CRC) Gal-4 is suppressed during the development of the disease [46-50], which indicates a role of Gal-4 as tumor suppressor. One of the challenges of studying galectins is that these lectins are soluble proteins with adhesive properties, which could have different functions depending on their localization (cytoplasmic, nuclear or extracellular), carbohydrate binding properties, and availability of their carbohydrate ligands. Therefore, the effects of Gal-4 could have divergent outcomes in cancer biology. Whereas most of the galectins share binding specificity towards poly-N-acetyllactosamine (poly LacNAc), they also possess specific glycan-binding profiles. Gal-4 opposing N- and C-terminal CRDs have different glycan-binding capacity, one recognizing sulfated glycan structures such as 3'-O-sulfation of short O-glycans [51], and the other can also bind with high-affinity to human blood group ABH sugars [52, 53]. Extracellular Gal-4 can mediate cell-cell and adhesion between cells to the extracellular matrix (ECM) which is composed of extracellular molecules secreted by cells that provide structural and biochemical support to the surrounding cells. In addition, Gal-4 may modulate intracellular processes such as protein transport.

Here we established a role of Gal-4 in cell invasion, migration and metastasis in PDAC (Chapter 2, Chapter3). Gal-4 was shown to inhibit metastasis formation by delaying migration of PDAC cells both in vitro and in vivo using zebrafish (Danio rerio) as an experimental model (Chapter 2). In addition, in human primary PDAC cells from patient material, high Gal-4 protein expression was negatively associated with migratory and invasive ability of these cells (Chapter 3). These results were also found in other kinds of cancer like colorectal [47] and hepatocellular (HCC) carcinomas [45], where Gal-4 also incites suppression of migration and motility of cancer cells. We showed that in PDAC patients, high Gal-4 expression was associated with reduced lymph node metastasis (Chapter 3). Analysis of Gal-4 expression in PDAC tissues showed high expression of Gal-4 in 80% of patients without lymph node metastasis, whereas 70% of patients with lymph node metastases had low Gal-4 expression. In HCC, Gal-4 expression was significantly lower in early/metastatic HCC patients, compared to the non-recurrent/metastatic HCC patients [45]. Low Gal-4 expression was associated with larger tumor size, angiogenesis, malignant differentiation, more advanced TNM stage (classification of malignant tumors) and poor prognosis. In human breast cancer, high levels of Gal-4 transcript correlate with better relapse-free survival [54]. This is also found in sinonasal adenocarcinoma [55] and in ileal carcinoids [56]. In several studies, Gal-4 is much higher expressed in the most less developed tumors compared to normal tissues [45, 54-56], and much lower expression was observed in the more advanced stages of non-intestinal adenocarcinoma or metastatic tumors. Thus, the level of Gal-4 expression correlates with developmental status of different cancers [55, 56].

In conclusion, our data indicate that Gal-4 has an adverse effect on cancer cell migration and metastasis. In addition, Gal-4 appears to be more expressed in earlier stages of metastasis formation than in later stages (Chapter 3, [45, 47, 48, 55, 56]).

1.1 Gal-4 inhibits Wnt signalling

We showed in this thesis that Gal-4 acts in the cytoplasm of the cells to inhibit cell migration and metastasis (Chapter2, Chapter 3). Activation of an important carcinogenic pathway, the Wnt/β-catenin pathway, has been associated with PDAC, being essential for initiation and progression of the cancer due to nuclear translocation of β-catenin [57]. β-Catenin is found mostly at adherens junctions in complex with E-cadherin and α-catenin, involved in cell-cell adhesion complexes [58]. These complexes are required for structural maintenance, function and cell polarity, which ultimately helps to control cell growth, creating and maintaining epithelial cell layers, and therefore be an obstacle for metastasis. The Wnt signalling pathway passes information from extracellular signals through cell surface receptors to the inside of the cell. Opposite to cell-cell adhesion complexes, Wnt pathway regulates gene transcription through free β-Catenin leading to cell proliferation and migration in cancer, hence helping the tumour to thrive. The dysfunction or disruption of this complex is a hallmark in the epithelial-to-mesenchymal transition (EMT), the most important and visible pro-carcinogenic transformation of a healthy cell to an abnormal cell.

Non-bound free β-catenin is targeted for destruction in healthy cells. When β-catenin is not fully destroyed it migrates into the nucleus and activates the Wnt signalling [59], which happens in many tumours.

Gal-4 was recently shown to display tumour suppressive effects via its ability to interact and decrease the Wnt signalling (Chapter 3, [47]). Using primary PDAC and CRC cells, cytosolic Gal-4 was found to interact and reduce free β-catenin levels (Chapter 3, [47]). Further, Gal-4 sensitized cells to a Wnt signalling inhibitor (Chapter 3), indicating that cells are more sensitive to induced cell death by this inhibitor, which is important for therapy effectiveness. In conclusion, our data in chapter 2 and 3 support a role of Gal-4 as a tumour suppressor in PDAC, by inhibition of Wnt signalling via reduction of β-catenin levels. This mechanism in turn may contribute to the Gal-4 induced inhibition of cell migration/metastasis (chapter 2, 3).

1.2 Gal-4 as an adhesion molecule

Molecular mechanisms by which Gal-4 acts as an adhesion molecule, between tumour cell-cell and/or tumour cell-extra cellular matrix (ECM), may partly explain the effects on cell migration and metastasis suppression. The two carbohydrate recognition domains (CRDs) in Gal-4 have different affinities, the C-terminal CRD binding with higher affinity to 3-sulfoglycans and the N-terminal CRD interacting preferably with blood group A and B antigens [51, 53]. We showed that Gal-4 expression is predominantly found on more in the initial stages of (differentiated) tumour cells (Chapter 2, Chapter 3, [55, 56]). We showed that Gal-4 was mostly found at the membranes between neighbouring cells in PDAC cells expressing endogenous Gal-4. These cells also showed an elevated surface binding capacity for Gal-4, indicating the presence of carbohydrate ligands for Gal-4 on their surface, whereas PDAC cells not expressing Gal-4 showed no Gal-4 binding ligands on their surface (Chapter 2). In chapter 6 we show that the Gal-4-binding PaTu-S cells express ABO blood groups ligands on their surface, which could facilitate Gal-4 binding. A switch in glycosylation, including loss of ABO antigens, associated with enhanced expression of sialic acids (Chapter 5) and short O-glycans (chapter 5) may result in loss of apical localization of glycoprotein rich in O-glycans (mucins). This in turn, could lead to the disturbance of cell-cell and cell ECM interactions [58, 60, 61]. Therefore, suppression of Gal-4 expression, in combination with over expression of mucins (Chapter 4) and a switch in glycosylation, may contribute to metastasis in PDAC and CRC by facilitating the release of cancer cells into the circulation.

In summary, we propose that Gal-4 may facilitate tumour cell-cell and tumour cell-ECM adherence. This could prevent release of tumour cells from the primary tumour, diminishing therefore migration and metastasis. Detailed structural identification of physiological and pathological ligands for Gal-4 could give us more insight in the molecular mechanisms by which Gal-4 acts in adhesive contacts in PDAC, and would provide leads for the development of tools to establish a role of Gal-4 in tumour cell adhesion.

2. Glycosylation in cancer

Within the immune system, the recognition of aberrant glycan structures on altered and aberrant cells by lectins of the immune system can lead to modified behaviour of immune cells and cancer progression. Therefore, the glycosylation patterns on CRC tissues (Chapter 4) and PDAC cells (Chapter 5) were determined, in relation to their binding to immune lectins. We showed pronounced differences in glycosylation profiles between CRC tissues and normal colon (Chapter 4) and between the PDAC tumour-like PaTu-S and metastatic PaTu-T cell lines, which show opposite characteristics in morphology and metastatic behaviour (chapter 5). The observed glycosylation profiles will have functional impact on cancer development.

In Chapter 4 we identified glycan structures on two tumour glycoproteins in tissue samples of 48 CRC patients. In tumor tissue we found an increased expression of specific glycans on Carcinoembryonic antigen (CEA), which contains mainly *N*-glycans and/or glycoprotein mucin MUC1 containing innumerous *O*-glycans. CEA and MUC1 are recognized by the glycan binding C-type lectins the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and the macrophage galactose-type lectin (MGL), respectively, compared to normal colon tissue. C-type lectins are important since they are present on the surface of immune cells, including dendritic cells and macrophages, and regulate important immune responses, such as activation or tolerance against antigens. In addition, CEA and MUC1 are known cancer markers, and an increase in serum levels of these proteins is associated with poor prognosis. In PDAC, we found pronounced differences in glycosylation profiles between the metastatic Pa-Tu-8988T (PaTu-T) and the tumour-like Pa-Tu-8988S (PaTu-S) cells (Chapter 5). In particular, we showed that the tumour-like PaTu-S cells displayed a higher expression of terminal fucose and an abundance of short *O*-glycans (Tn antigens, GalNAcα1-*O*-Serine/Thrionine glycan), probably due to an elevated initiation of O-glycosylation. This coincided with higher binding of MGL and Gal-4. By contrast, the surface of the metastatic PaTu-T cells contained relatively high levels of oligomannose-type structures, and elevated levels of sialic acids, specially α2,3-linked *N*-acetylneuraminic acid (NeuAc). In conclusion, the disparity of these glycan profiles on both cancer types could result in dis-

tinct immune responses via the aberrant interaction with different immune-related lectins (Chapter 4 and Chapter 5), but may also affect metastatic properties, which may be an important subject for further studies.

3. Modulation of Glycosylation by Hypoxia Inducible Factor

After being initiated and extended to form branches, glycans can be terminated by a fucose or a sialic acid (in general *N*-acetylneuraminic acid (Neu5Ac)), such as in ABO and Lewis blood group antigens, by the action of fucosyltransferases or sialyltransferases, respectively [22]. Modifications in fucosylation and/or sialylation in cancer usually result in altered expression of the Lewis blood group family of antigens, particularly Lewis X (Lex), Lewis Y (Ley) and/or ABO blood group antigens, and these alterations cause changes in functional properties of the tumour cells. Nevertheless, it remains mostly unknown which factors are responsible for directing the alterations in glycosylation in cancer. An important process which induces alterations early in cancer development is the low oxygen level (hypoxia) within tumours. Cancer cells undergo distinct metabolic changes to cope with their hypoxic environment. These changes are achieved at least partly by the action of transcription factors called hypoxia-inducible factors (HIFs). Transcription factors regulate gene expression to produce mRNA and ultimately proteins. In Chapter 6 we describe that the α1,2-fucosyltransferase genes *FUT1* and *FUT2* are highly expressed in tumour-like PaTu-S cells, whereas expression is hardly detected in metastatic PaTu-T. In Chapter 6 we show that expression of *FUT1* and *FUT2* is regulated by the hypoxia-induced transcription factor 1α (HIF-1α). HIF-1α inhibits the expression of both *FUT1* and *FUT2* in the metastatic PaTu-T cells, thereby lowering the expression of α1,2-fucosylated structures on the surface of PaTu-T cells. In conclusion, our results indicate that in PDAC α1,2-fucosylation, including the synthesis of blood group A and B antigens, is regulated by HIF-1α. These data contribute to an increased understanding of how the expression of glycans can be modulated in the developing primary tumour.

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